THE IMPACT OF IMMUNOSUPPRESSION ON THE DURATION AND LEVEL OF FECAL SHEDDING OF E. COLI O157:H7 IN CALVES

by

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Abstract

Escherichia coli O157:H7 has emerged as a significant human food-borne pathogen over the past two decades. While cattle have been identified as the major reservoir of the pathogen, the dynamics of shedding are still largely unknown. The role immunosuppression may play on fecal shedding in cattle is explored.

The first study determined whether immunosuppression induced by dexamethasone injections affects the level and duration of fecal shedding of E. coli O157. Six one week old Holstein bull calves were injected intramuscularly with dexamethasone and orally inoculated with 109 CFU of a mixture of three nalidixic-acid resistant strains of E. coli O157. Another five one week old Holstein bull calves, only inoculated with the E. coli O157, served as controls. All calves were necropsied and samples from the gastrointestinal tract were cultured. Dexamethasone treated calves shed at higher levels on days four and seven post-inoculation, but not thereafter. The data from this study suggest that there may be a time dependent correlation between dexamethasone immunosuppression and the concentration of E. coli O157 an animal will shed in the feces and that transient immunosuppression does not result in prolonged shedding of E. coli O157.

The goal of the second study was to determine whether calves immunosuppressed by persistent infection with bovine viral diarrhea virus (BVDV) will shed E. coli O157 at a higher level and for a longer duration than a normal animal. Nine six to eight week old calves persistently infected with non-cytopathic BVDV and eight normal calves obtained from separate cow-calf operations were orally inoculated with 109 CFU of a mixture five nalidixic-acid resistant strains of E. coli O157. All calves were necropsied and samples from the gastrointestinal tract cultured. There was no statistical difference in the concentration of E. coli O157 shed or the duration of shedding between the persistently infected BVDV calves and the control calves throughout the length of the study. The data suggest that immunosuppression caused by persistent infection with non-cytopathic

BVDV infection does not play a role in the level or duration of shedding of E. coli O157:H7 in calves.

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CHAPTER 1 - A Literature Review of *Escherichia coli* O157:H7 Fecal Shedding Patterns in the Bovine

Introduction

According to the Centers for Disease Control (CDC), Escherichia coli O157:H7 causes about 73,000 human illnesses annually in the United States; of which approximately 2,000 result in hospitalization and 60 result in death (Frenzen, et al, 2005). Although Escherichia coli is a normal gastrointestinal inhabitant of mammals, there are several strains that are pathogenic. In 1982 a new pathogen was identified with two outbreaks of severe bloody diarrhea associated with a fast-food chain restaurant. This new pathogen was a strain of E. coli that expressed the somatic antigen (O) 157, the flagellar antigen (H) 7, and toxins similar to Shigella dysenteriae (Armstrong, et al, 1996). E. coli O157:H7 has become a pathogen of significant public health concern, since it was first identified over two decades ago.

Asymptomatic cattle have been identified as the primary reservoir of E. coli O157. For the most part, humans become infected with E. coli O157 by consuming food or water contaminated with feces, but also through direct contact with infected animals or humans (Armstrong, 1996). Human illness can range from transient diarrhea to severe bloody diarrhea, or secondary complications leading to death. It has been estimated that the annual cost of E. coli O157 illness in the United States is $405 million, including medical costs and lost productivity (Frenzen, et al, 2005). Therefore, it is important to evaluate the epidemiology of this organism to determine means of reducing the risk of human infection.

Epidemiology of E. coli O157

Humans

According to the Center for Disease Control's (CDC) data from 10 states, in 2006 there were 17,252 laboratory-confirmed cases of food borne illnesses. Of these, 590 were due to Escherichia coli O157:H7 (MMWR, 2007). In the 1996 FoodNet data from five states, of the 8,576 laboratory-confirmed cases of food borne illnesses, 340 were due to E. coli O157 (Wallace, et al, 2000). Therefore, there has not been a significant decrease in the number of cases of illness due to E. coli O157 in the last decade. This may be due to the fact that the number of sampling sites has doubled over the decade, there may be more awareness of the pathogen among laboratory sites (and more tests are performed to look for the pathogen in samples), and in 2001 the CDC introduced a new model to assess trends. The CDC reports that there was a significant decline in cases from 2002 to 2004, most likely due to the implementation of Hazard Analysis and Critical Control Point (HACCP) protocols at slaughter plants by the United States Department of Agriculture – Food Safety and Inspection Service in 2002. However, there has been an increase in reported E. coli O157 infections since 2005, primarily associated with lettuce and bagged fresh spinach (MMWR, 2007).

E. coli O157 is transmitted to humans by contaminated food and water consumption, from infected animal to person, and from infected person to person. Approximately 85% of E. coli infections are due to ingestion of contaminated foods (Frenzen, et al, 2005). In the United States, ingestion of undercooked ground beef has played a major role in outbreaks. Other food borne sources of E. coli O157 include roast beef, salami, mayonnaise, unpasteurized apple juice, unpasteurized milk, cantaloupe, grapes, alfalfa sprouts, potatoes, lettuce, and spinach (Armstrong, et al, 1996; MMWR, 2007; Nataro and Kaper, 1998; Rangel, et al, 2005). Waterborne outbreaks have also been identified involving ingestion of drinking water or presumably swallowing of water during swimming in freshwater areas (Armstrong, et al, 1996; Rangel, et al, 2005). Outbreaks associated with animal contact are a newly recognized source of transmission. Contact with hide or environment contaminated with feces is the most likely mode of fecal-oral transmission (Keen, et al, 2006; Rangel, et al, 2005).

Infection with E. coli O157:H7 causes three primary syndromes in humans: hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura, as well as a variety of secondary clinical complications (Bach et al, 2002; Nataro and Kaper, 1998; Park et al, 1999). Symptoms of hemorrhagic colitis include abdominal cramps, bloody diarrhea, and dehydration lasting from two to nine days. Most illnesses are self-limiting but can become severe in children, elderly, and other immunocompromised individuals. Hemolytic uremic syndrome (HUS) symptoms include hemolytic anemia, thrombocytopenia, and acute renal failure. 85-95% of severe hemolytic-uremic syndrome in children in the United States is caused by E. coli O157:H7. Thrombotic thrombocytopenic purpure (TTP), in contrast to HUS, occurs in adults and involves neurological signs in addition to the hemolysis, thrombocytopenia, and renal failure (Armstrong et al, 1996; Bach et al, 2002; Nataro and Kaper, 1998; Park et al, 1999).

Non-bovine and Bovine

Although cattle are thought to be the most important reservoir for E. coli O157:H7, other animal reservoirs have been identified. In one study, fecal samples from several different farms were collected and E. coli O157 was detected from a horse, dogs, pooled bird samples, and pooled fly samples (Hancock et al, 1998). Sanderson et al (2006) were also able to collect positive samples from bird feces and flies. Doane et al (2007) collected positive samples from swine, turkeys, and chickens. One other study was able isolate E. coli O157 from wild bird feces (primarily gulls) collected along a shoreline and an urban landfill in England (Wallace et al, 1997). Another study detected E. coli O157 in the feces of free ranging sheep (Kudva et al, 1996). Free range deer have also been found to shed E. coli O157 in feces (Fischer et al, 2001; Sargeant et al, 1999). Over a one year period, Chapman et al (1997) were able to culture E. coli O157 from sheep and swine rectal fecal samples. While these studies indicate that E. coli O157 lacks host specificity, few animals other than cattle have been studied enough to determine their role in the spread of this human pathogen.

Since non-bovine sources have been identified as a source of E. coli O157, environmental sources have also been investigated. Water samples have tested positive

for O15 in studies at the farm and feedlot levels (Hancock et al, 1998; Van Donkersgoed et al, 2001; Sanderson et al, 2006). Van Donkersgoed et al (2001) and Sanderson et al (2006) were able to obtain positive cultures from feed in feedbunks. Dodd et al (2003) sampled feed samples from feed bunks in 54 feedlots and found a prevalence of 14.9%. In a longitudinal study, environmental samples in a Kansas feedlot had a 0.7% prevalence of the bacteria prior to cattle entry. The environmental prevalence increased significantly to 17.1% after arrival of cattle, and positive samples were able to be obtained from lagoon water, bunk water, pen surfaces, pre-access and post-access feed at some point in the 13 week sampling time (Sanderson et al, 2006). In contrast, Hancock et al (1998) tested feed samples prior to being placed in the bunks and did not obtain any positive samples. One study examined how the moisture and manure content in feedlot pens affect the persistence of E. coli O157 in the environment. The conclusion was that as long as there was more than five percent manure on the ground of the pen with some amount of water, the bacteria either increased or persisted over the two week study period (Berry and Miller, 2005). These studies suggest that fecal contamination of the environment by cattle, and/or other animals (less likely), is important in the persistence of E. coli O157 in the environment and transmission among animals.

Many studies have been done to determine the farm and feedlot prevalence of E. coli O157 among cattle in the United States. In a recent longitudinal study of four farms, 3.4% of dairy cattle were determined to be shedding E. coli O157 (Doane et al, 2007). This is in contrast to an earlier study which determined a dairy herd prevalence of 1% (Hancock et al, 1997a). Doane et al (2007) also determined a prevalence of 4.7% in four beef herds, which is higher than the 1.9% (range of 0.7-2.3%) prevalence that Sargeant et al (2000) found. In a one time sampling of 15 herds from five different states, an average of 7.4% of beef calves at weaning time were shedding E. coli O157 in their feces (Laegreid et al, 1999). Sampling from 100 feedlots in 13 states, Hancock et al (1997b) found the prevalence to be 1.8% among animals, while a more recent study of 73 feedlots in four states had a 10.2% prevalence (Sargeant et al, 2003). Outside of the United States, there was a 1.9% prevalence in three Australian dairy herds (Cobbold and Desmarchelier, 2000), a decrease from 10.6% in 1998 to 5.6% in 2000 on a Japanese dairy (Ezawa et al, 2004), and an overall prevalence of 4.2% among different types of cattle herds in

England and Wales (Paiba et al, 2003). This prevalence data indicates that there is a similar distribution in several countries. Studies obtaining fecal samples at the slaughter plant had a prevalence that ranged from 7.5% in England (Omisakin et al, 2003) to 13% in Australia (Fegan et al, 2004). These studies indicate that E. coli O157 is present on nearly all cattle operations and is therefore ubiquitous among cattle populations.

Factors Affecting Shedding Levels in the Bovine

In order to better understand the epidemiology of E. coli O157:H7 in bovine populations, the duration and magnitude of fecal shedding, locations of possible persistent colonization within the gastrointestinal tract, as well as other factors need to be understood so that animals that could pose a greater risk of shedding can be identified.

Duration of Fecal Shedding

Several studies have been done where animals were individually inoculated with previously isolated E. coli O157 strains. Cray and Moon (1995) inoculated pre-weaned 3-14 week old calves and adults with 1010 CFU and found them to be shedding for up to 14 weeks post inoculation. Sanderson et al (1999) found that 1 week old calves inoculated with 108 CFU shed for a shorter period of time (20-43 days post inoculation) with the day to day shedding levels being variable. Weaned calves shed for a similar duration when inoculated with 1010 CFU and had a significant decrease in the magnitude shed during the first two weeks post-inoculation (Brown et al, 1997). Another study also looked at weaned calves but gave a significantly lower dose of inoculums (<300 and <1000 CFU) resulting in calves shedding transiently for at least 14 weeks (Besser et al, 2001). A study in which 22 adult cattle were orally inoculated found three distinct patterns of shedding: those that stopped shedding by one week, those who shed for about one month (the majority of the animals), and those animals that shed for two months or longer. As in previous studies, all animals shed consistently for 7-10 days post inoculation and intermittently afterwards with no difference in shedding levels between the three groups (Grauke et al, 2002).

Other studies have looked at naturally infected herds over a time period. One study found considerable variability in shedding among dairy farms that were tested

monthly for a year, with some herds having frequent positive samples and others rarely having positive samples. Overall, 63% of the animals shed E. coli O157 in the feces for less than one month (Besser et al, 1997). Khaitsa et al (2003) collected fecal samples weekly for 19 weeks, beginning at the time of arrival to the feedlot, and found three distinct phases in fecal shedding. The first eight weeks were termed the pre-epidemic period where there was a low pen prevalence in which infected cattle were shedding for a short duration (about two weeks). This was followed by an epidemic period (9-15 weeks post arrival), during which the pen prevalence was high and cattle shed for an average of four weeks. The final post-epidemic period (16-19 weeks post arrival) had medium pen prevalence and shedding duration decreased. 30% of the cattle were still shedding at the end of the study with a mean duration of three weeks. In contrast, Sanderson et al (2006) collected feedlot fecal and environmental samples prior to cattle entry and twice weekly for 11 weeks after cattle entry and obtained a significantly high pen prevalence three weeks after cattle entry.

Gastrointestinal Tract Colonization

Identifying the location of E. coli O157 is important in determining if there is a specific place where the bacteria replicate or persist in the bovine. Early studies narrowed the primary site of colonization to the proximal gastrointestinal tract. Specifically, one study found all weaned calves having positive cultures from the rumen and omasum and determined the primary site of localization as the forestomachs and proliferation of the bacteria within the ingesta (Brown et al, 1997). A later study found that while samples can be identified in the upper gastrointestinal tract, E. coli O157 does not stay there for long. It moves out of the duodenum within one hour of inoculation with 1010 CFU of E. coli O157 and is present in the feces within the colon within six hours post-inoculation (Grauke et al, 2002). Samples from slaughterhouse sampling found that detection in the colonic contents is more frequent than in the rumen. Furthermore, E. coli O157 in the rumen was associated with the rumen wall (Lave et al, 2003). This may suggest that the rumen wall may be a site of colonization, but the bacteria are cleared quickly from the rumenal contents and into the distal gastrointestinal tract, where it is detected with fecal sampling. Weaned calves that are fasted for 48 hours prior to inoculation and also receive

an immunosuppressive dose of dexamethasone had positive samples in the gallbladder (Stoffregen et al, 2004). Other studies have found positive samples in the gallbladder at slaughter, suggesting another possible site of colonization (Reinstein et al, 2007).

Of more interest in the past few years has been the detection of E. coli O157 from a lymphoid rich region near the recto-anal junctional area (RAJ). This area was first identified by Naylor et al (2003) in weaned calves that were inoculated with 109 CFU and had been consistently shedding for at least 14 days post inoculation. All calves had positive fecal samples and high bacterial counts from colon and midrectum samples. In addition, five tissue samples proximal to the RAJ and one tissue sample at the RAJ were taken for testing. In 13 of the 15 animals that were still shedding two weeks after inoculation, the tissue samples immediately proximal to the RAJ had high bacterial counts. These animals also had higher bacterial counts in the feces compared to contents collected from elsewhere in the gastrointestinal tract. Further research has been conducted examining this RAJ location. Fecal samples and mucosal swab samples taken from 1cm and 15cm proximal to the RAJ immediately after slaughter indicated that high mucosal carriage (indicated as > 103 cfu/swab) were associated with high fecal concentration (indicated as > 103 cfu/g) (Low, et al, 2005). Another study obtained samples from three animals (two inoculated with 1010 CFU and one naturally infected) that had been consistently shedding E. coli O157 for at least two months. All three animals had positive recto-anal junction mucosal swab (RAMS) samples, however only one of the three animals had a positive fecal sample (Lim et al, 2007). Since there were two samples in this study that tested positively from the RAMS and not in the feces, it was suggested that RAMS is a more reliable technique for testing for E. coli O157 than fecal sampling. Cobbold et al (2007) collected RAMS and fresh fecal samples from a feedlot over a 14 week period and also found that RAMS samples were more sensitive than fecal sampling.

Age

Among animals that have been inoculated with the same dose, calves shed significantly higher numbers and for a longer time period than adults (Cray and Moon, 1995). Among naturally infected calves of 1-10 months of age, calves that are 5-6 months

of age have a higher prevalence of shedding than the other ages (Widiasih et al, 2003). Hancock et al (1997a) determined that among dairy herds, weaned heifers had a higher prevalence than adults. Weight can be correlated with age in cattle and Dargatz et al (1997) found that among cattle entering the feedlot, pens that contained animals that weighed less than 700 pounds were more likely to culture positive for E. coli O157.

Season

Several studies have examined the prevalence and shedding levels of E. coli O157 compared to the time of year but with conflicting results. Besser et al (1997) found that US dairy cattle shed less during the winter and spring and higher levels in the fall and summer. Smith et al (2005) also found higher prevalence during the summer than during the winter in US feedlots. In England and Japan, the highest shedding levels were also during the spring and summer (Chapman et al, 1997; Mechie, et al, 1997; Widiasih, et al, 2003). Whether these seasonal changes are due to ambient temperature, moisture content, insect population, or other factors is unknown. Edrington et al (2006) found a higher correlation between length of daylight and prevalence (r=0.67) than temperature and prevalence (r=0.43). Using this hypothesis, a test group of commercial cattle were exposed to extended hours of light via artificial lighting. Only on one day did the test group have a higher fecal prevalence than the control group. Edrington et al (2007) also hypothesizes that the hormone triiodothyronine (T3) may play a role in the seasonal shedding patterns of E. coli O157 in cattle. However, further studies need to be done to replicate the findings.

In contrast to the above studies, samples collected from a Scottish slaughter house found a higher prevalence during the cooler months when cattle are housed inside (11.2%) than during the warmer months (7.5%) (Ogden et al, 2004), suggesting that animal to animal contact in high density situations may play a role in the seasonal prevalence. Two studies conducted in the United States indicate that shedding is not season dependent. Samples collected from a Kansas feedlot from the middle of August to the end of February had the highest shedding prevalence in February and the second highest in August with no significant differences overall (Alam and Zurek, 2006). Another feedlot study conducted in North Dakota found the highest fecal prevalence in

March (Khaitsa et al, 2006). In this study, upon arrival to the feedlot in October, the fecal prevalence was 1.4%, which rose significantly to 6.9% 28 days later. The highest prevalence was 53% in March, which dropped in April to 21%. There were some management changes (rearrangement of animals into pens by weight and change in diet from a growing to finishing diet) between the November and March samplings which may have affected the prevalence more than season itself. However, the pens were also the muddiest during March due to melting of snow and ice, which could also have contributed to the greater prevalence. This is in agreement with Smith et al's (2005) finding that the probability of a pen testing positive for E. coli O157 in the winter months was associated with the pen being wet and muddy.

Type of Diet

Two month old calves fed high roughage diets do not shed at higher levels or for longer than those fed a high concentrate diet and the rumenal populations of E. coli O157 are no different in either group (Tkalcic et al, 2000). However, adults fed a complete hay diet (alfalfa or grass) shed for a longer duration than those fed a primarily grain diet (Hovde et al, 1999). The difference in findings between calves and adults may be due to the fact that calves do not have a fully developed and functioning rumen until about four months of age and are therefore unable to digest feeds in the same manner as adults. After examining 100 feedlots from 13 states, Dargatz et al (1997) determined that feedlot pens were more likely to culture positive for E. coli O157 if the feed contained barley (P=0.003), and pens were less likely to be positive if feed contained soybean meal (P=0.041). Buchko et al (2000) fed three different diets to three groups of yearling steers and inoculated them with 1010 CFU of an E. coli O157 strain and collected fecal samples for 10 weeks. The corn diet was 85% cracked corn and 15% alfalfa silage. The cottonseed and barley diet consisted of 15% whole cottonseed, 70% barley, and 15% alfalfa silage. The barley diet was 85% barley and 15% alfalfa silage. The steers were acclimated to the diets prior to inoculation. On days five and seven the corn group shed significantly lower levels of E. coli O157 than the other two groups. On day 49, both the corn and cottonseed with barley groups shed significantly less than the barley group and on day 63 the cottonseed with barley group shed less than the barley group. Overall, the

barley group had significantly more positive samples than the corn group ($P<0.005$) and the cottonseed with barley group ($P<0.05$). Although there was no difference in shedding levels between the groups on most days, Buchko et al determined that cattle fed a diet of primarily corn shed E. coli O157 fewer days than if fed primarily barley. A more recent study compared the effect canola oil added to either a barley or corn based diet would have after inoculation with E. coli O157 (Bach, et al, 2005). One group was fed a barley based diet with no canola oil, a second group was fed the same barley based diet with six percent canola oil, a third group was fed a corn based diet with no canola oil, and the fourth group was fed the same corn based diet with six percent canola oil. Adding the oil to the feed had no effect on shedding levels. Although there was no overall difference in shedding levels among the four treatment groups, there were significantly more positive samples obtained from the barley only group than the other three groups. These studies suggest that feed type may have an effect on shedding levels, but the reasons for the difference are still not understood.

Stress

Cattle encounter stressful situations at various points in their life. During these time periods, there are immunological changes that occur in the body that allow them to be more susceptible to various infections which may lead to the increased shedding of organisms.

Weaning in beef cattle often occurs between three and six months of age. In a longitudinal study examining 14 herds, Hancock et al (1997a) found that weaned heifers had a higher prevalence than unweaned calves or adults. Fasting can occur among cattle during the weaning period where the calf is transitioning from milk to roughage and/or grain diets as well as during transport. One week old calves that were fasted prior to and after a second inoculation and fasted after a third inoculation showed no difference in shedding levels (Sanderson et al, 1999). Weaned calves that are fasted after inoculation also show no difference in shedding levels in the feces or rumenal proliferation (Brown et al, 1997; Harmon et al, 1999). In another study, there was no increase in shedding among three to four month old calves that were inoculated prior to having food withheld for 24 hours. In contrast, if food was withheld for 48 hours prior to inoculation, they shed at

significantly higher levels (P=0.001) than those calves that had access to feed (Cray et al, 1998).

Several studies show that shipping of cattle does not increase E. coli O157 fecal shedding in cattle. Barham et al (2002) found that short term shipping of steers and heifers on trailers (30-40minutes) had no effect on shedding. Minihan et al (2003) followed cattle from the farm to the slaughter plant and found that the prevalence of E. coli O157 shedding in cattle sampled at the farm, post-transport to the slaughter facility, and once inside the slaughter facility was 18%, 13%, and 12% respectively. Carcasses were swabbed immediately following slaughter, and no positive samples were obtained. This study indicates that good hygienic practices at the slaughter facility are important in preventing food contamination.

Bach et al (2004) found that non-preconditioned steer calves hauled for 15 hours did have a significantly higher level of E. coli O157 shedding, compared to preconditioned calves hauled 15 hours and compared to short haul calves (3 hours) that were either preconditioned or not preconditioned, suggesting that compounding stresses at one time, may cause an increase in shedding levels of E. coli O157.

The prevalence of E. coli O157 was significantly lower in lactating dairy cows than in non-lactating cows, calves and heifers in one study (Mechie et al, 1997). This study also found that shedding levels in lactating dairy cattle were highest during the first month post-partum, fell during the rest of lactation prior to another peak seven months into lactation. In contrast, Edrington et al (2004) found that stage of lactation had no effect of the overall prevalence of dairy cows shedding E. coli O157.

Re-infection / Re-inoculation

Studies examining the effect re-inoculation of calves or adults with a similar or lower dose of E. coli O157 showed that the re-inoculated animals shed for a shorter duration or for about the same duration as those animals inoculated only one time (Cray and Moon, 1995; Grauke et al, 2002; Sanderson et al, 1999).

Definition of a "Super-shedder"

The term "super-shedder," has been coined for animals that shed E. coli O157 at higher levels and for a longer duration of time compared to other animals in the same location (Matthews et al, 2006). These animals may be important in the on-farm epidemiology, ecology, and shedding of E. coli O157 (Omisakin et al., 2003). As such, they may also play an important role in pre-harvest food safety. Modeling data suggests that the majority of transmission within a farm may be the result of a small number of these "super-shedders" resulting in elevated prevalence (Matthews, et al 2006). Therefore, determining the existence and what causes these "super-shedders" to shed at higher levels and/or for a longer duration is important. However, there are inconsistencies in the literature regarding the definition of what a "super-shedder" animal is. The thresholds for high level and long duration have seemingly been arbitrarily chosen in different studies.

Besser et al (2001) classified calves that shed at greater than 1000 cfu/g feces as shedding high levels in their low dose inoculation study and noted marked variability in the level of shedding. When examining fecal versus RAMS samples for shedding, Low et al (2005) classified high shedders as animals that had greater than or equal to 103 cfu/g feces, whereas Naylor et al (2003) and Cobbold et al (2007) set the cutoff at 104 cfu/g feces.

Lim et al (2007) determined shedding for greater than two months as a long duration of shedding. Naylor et al (2003) determined animals that shed for at least two weeks as being persistently infected. This definition seems inappropriate, since the majority of studies suggest fecal shedding of up to one month as being normal in either inoculated or naturally infected animals, and the majority of inoculated animals shed for greater than two weeks. Cobbold et al (2007) defined persistent shedding as having greater than 4 consecutive RAMS positive samples over a 14 week testing interval.

Besser et al (1997) concluded that although fecal shedding is transient and of short duration in the majority of individuals, among herds that have high prevalence, there may be more likelihood of recurrent exposure leading to persistent shedding. Therefore, it needs to be determined whether some animals persistently shed E. coli O157 because they remain colonized or because they are more susceptible to re-colonization.

Prevention and Control Methods

Effectively reducing the fecal shedding of E. coli O157 in live-cattle would result in fewer human infections from contaminated foods according to a simulation study (Jordan et al, 1999). Ideally, this would be accomplished via two strategies: by decreasing the number of animals that shed the organism and by decreasing the number of organisms shed by an individual animal or a group of animals. Since the feces have been found to be a primary source of spread among cattle, proper sanitary management of the environment is of primary importance. Smith et al (2002) found that while scrubbing and disinfecting water tanks with chlorine substantially decreased the number of coliform bacteria in the tank initially, 24 hours after cleaning, bacterial counts were be back to pre-cleaning levels. Therefore, while proper cleaning methods are important to animal husbandry, it can be labor intensive and time consuming, and may only result in short term management of fecal contamination.

Several studies have looked at alternative methods of reducing shedding of E. coli O157. Brashears et al (2003b) isolated several strains of lactic acid bacteria from the feces of cattle not shedding E. coli O157. Of all the strains isolated, 52% of the lactic acid bacterial isolates were able to inhibit E. coli O157 growth on media and significantly inhibited growth in colonic samples and rumen fluid in vitro. Two of the isolates, Lactobacillus acidophilus or Lactobacillus cristatus were then placed into the feed and water of cattle in a feedlot. Over time, animals that were given the Lactobacillus acidophilus were 49% less likely to shed E. coli O157 in the feces compared to a control group. At slaughter, hide samples were taken and animals that received either microbial agent had significantly lower hide prevalence than the control animals (Brashears et al, 2003a).

Rather than examining the use of competitive bacteria as a method of decreasing shedding in an animal, Dziva et al (2007) used a mutant form of E. coli O157 to examine whether colonization would be affected. Three 14 day old bull calves were inoculated with a wild type and three others were inoculated with a strain that had the EspA gene deleted. This gene allows the bacteria to form filamentous attachments to the gastrointestinal cells of the host. Results showed that animals that received the mutant strains shed significantly lower levels in the feces from day one post-inoculation and for a

shorter duration than the control animals. However, in the follow-up vaccination trial, when a purified version of the mutant strain was given three times intramuscularly and intranasal once to 14 day old bull calves, there was no difference in the levels or duration of shedding in the feces of vaccinated calves compared to the control calves.

Potter et al (2004) examined the effect a different type of vaccine has on shedding prevalence. Based on the determination that certain proteins (Tir, EspA, and EspB) secreted by the type III system play a role in gastrointestinal tract colonization of non-bovine hosts, vaccines were prepared using these proteins and given subcutaneously to cattle in three separate studies. In the first study, the test calves were vaccinated two weeks prior to inoculation with a 108 cfu dose of E. coli O157. Over a two week period, the vaccinated group shed less ($P=0.05$) than the control group that received an adjuvant. In the second study, the group of yearlings that received three doses of vaccines 49 days prior to inoculation shed less and for a shorter time than the non-vaccinated group. The last study showed that cattle receiving three doses of vaccine post-arrival to the feedlot shed less ($P=0.04$) than the control group.

In calves challenged orally and rectally with E. coli O157, rectally administered polymyxin B (an antibiotic) and chlorahexidine gluconate (an antiseptic agent) treated animals shed significantly lower levels ($P<0.001$) of E. coli O157 in the feces than the control calves, with no difference seen between the two treatment types (Naylor et al, 2007). A problem with this study was that animals were not evenly distributed into treatment groups, with significantly more being present in the chlorahexidine treatment groups.

Conclusion

E. coli O157 is ubiquitous among cattle populations in all aspects of the production system. Fecal shedding by cattle is currently thought to be the major route of spread to other cattle and to humans. However, there are still many factors that are not completely understood about the epidemiology of E. coli O157 in the bovine. One important aspect is being able to determine which animals are more likely to harbor the organism in their gastrointestinal tract and, consequently, are more likely to shed it.

Further research is needed to increase our knowledge about the dynamics of E. coli O157 in the individual animal and in the herd in order to reduce the chances of it contaminating human foods.

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CHAPTER 2 - Impact of Dexamethasone Induced Immunosuppression on the Duration and Level of Shedding of E. coli O157:H7 in CalvesChapter Title Here

Abstract

The goal of this study was to determine whether immunosuppression plays a role in the level and duration of fecal shedding of E. coli O157. Immunosuppression was induced in calves by administering dexamethasone. Six one week old Holstein bull calves were injected intramuscularly with dexamethasone and orally inoculated with 109 CFU of a mixture of three nalidixic-acid resistant strains of E. coli O157. Five one week old Holstein bull calves that were given the same oral inoculation of E. coli O157, but not the dexamethasone injections, served as controls. As expected, dexamethasone treatment induced a glucocorticoid leukogram on complete blood counts, confirming the immunosuppressive status in the dexamethasone calves. All calves were examined daily and fecal samples were collected three times a week for detection and enumeration of the nalidixic-acid resistant E. coli O157. Four weeks after the last calf stopped shedding, all calves were necropsied and samples from the gastrointestinal tract were taken for the detection of the nalidixic-acid resistant E. coli O157. Dexamethasone treated calves shed at higher levels (P value=0.038) on days four and seven post-inoculation, but not thereafter. No E. coli O157 was detected at necropsy. The data from this study suggest that there may be a time dependent correlation between dexamethasone immunosuppression and the concentration of E. coli O157 an animal will shed in the feces, that higher levels of E. coli O157 will be shed for as long as dexamethasone suppresses the immune system, and that transient immunosuppression does not result in prolonged shedding of E. coli O157.

Introduction

Enterohemorrhagic Escherichi coli O157:H7 has emerged as a pathogen of public health significance over the past two decades. Although infected cattle are asymptomatic, infection can cause hemorrhagic colitis in humans. The colitis can be more severe in children, the elderly, and other immunocompromised individuals. E. coli O157:H7 is also the cause of 85-95 percent of severe hemolytic-uremic syndrome in children in the United States (Armstrong, et al., 1996). Several studies have indicated relatively high herd prevalence among farm and feedlot cattle and beef and beef products have been identified as a significant human source for the pathogen (Hancock, et al, 1998; Galland, et al 2001).

Some researchers have proposed the presence of animals termed "super shedders," which shed E. coli O157 at higher levels and for a longer duration of time compared to other animals in the same location. These animals may be important in the on-farm epidemiology, ecology, and shedding of E. coli O157 (Omisakin et al., 2003). As such, they may play an important role in pre-harvest food safety. Modeling data suggests that the majority of transmission within a farm is the result of a small number of these "super shedders" resulting in elevated prevalence (Matthews, et al 2006). Therefore, determining what causes these "super shedders" to shed at higher levels and/or for a longer duration is important. Stoffregen, et al (2004) used dexamethasone immunosuppression in a study of weaned calves to determine the susceptibility of the gastrointestinal tract to colonization of E. coli O157. The goal of this study was to determine whether dexamethasone immunosuppression plays a role in the level and duration of fecal shedding of E. coli O157.

Materials and Methods

The basic outline of the study is illustrated in Figure 2.1.

Calf Management

Twelve two-day-old Holstein bull calves were obtained from a dairy and transported to a BL-2 facility at Kansas State University. Upon arrival, each calf was ear tagged and placed in a separate pen. One calf was euthanized one day after arrival due to poor and

declining health. Calves were randomly assigned to either the dexamethasone treatment or the control group and arranged in the BL-2 facility by placing all dexamethasone treated calves (n=6) in one room of the barn and all controls (n=5) in a separate room of the barn. Each calf was housed in an individual pen with individual feed and water buckets and contact between calves was not allowed. All calves were offered free choice water from day 1. Calves were initially bottle fed milk replacer without antibiotics, and within the first week, they were transitioned into drinking milk replacer from a bucket. Three weeks after their arrival, all calves were offered grain and free choice hay.

Pre-Challenge

Calves were acclimated to the facility for one week and fecal samples were obtained from the rectum once and tested for E. coli O157 to establish negative status according to the standard protocol in our lab (Alali et al., 2004). Briefly, a one gram fecal sample was incubated (37oC) for six hours in nine mL of gram negative broth (BD, Franklin Lakes, NJ) with vancomycin (8g/L), cefixime(0.05mg/L), and cefsulodin (10mg/L) (GN-vcc). The samples underwent manual immunomagnetic separation using the standard protocol, and were plated on Sorbitol MacConkey agar containing cefexime (50 ng/ml) and tellurite (2.5 g/ml) (SMAC-ct) and incubated overnight at 37 oC. A maximum of six colonies morphologically typical of E. coli O157 were selected, streaked on blood agar and incubated overnight. Indole and latex agglutination tests were performed, and those colonies that were positive on both tests were confirmed as E.coli with Rapid API kits (Biomerieux, Hazelwood, MO Three days prior to inoculation with E. coli O157, jugular vein blood samples were taken from each calf into heparinized blood tubes and CBCs were run to obtain baseline blood-work values.

Calf Challenge and Sampling Schedule

After acclimatization, all calves were inoculated with 109 CFU of nalidixic acid resistant E. coli O157 directly into the rumen using an esophageal feeding tube. The inoculum was prepared from a frozen stock of three strains (01-2-10004, 01-2-10567, and 01-2-10597) adapted to nalidixic acid (20 µg/ml). Each strain was grown individually, pooled together on the day of inoculation, and added to milk replacer to inoculate the calves. Treatment calves received dexamethasone (0.25mg/kg, IM) for two days before,

the day of, and two days after oral inoculation with the O157. Control calves did not receive dexamethasone injections. All calves were examined daily and fecal samples were collected three times a week (Monday, Wednesday, and Friday) for detection and enumeration of the nalidixic resistant strain of E. coli O157. Blood samples were collected from all calves into EDTA tubes and CBCs were run on days three, 14, 25, and 42 post E. coli O157 inoculation. Fifty-one days post- E. coli O157- inoculation (21 days after the last calf stopped shedding E. coli O157) all calves were necropsied and samples from the gastrointestinal tract (lingual tonsil swab, cranial lymph node swab, rumen contents, omasal contents, abomasal contents, jejunal contents, ileum contents, cecal contents, colon contents, rectal contents, recto-anal junction mucosal swab, mesenteric lymph node swab, peyers patches swab, gallbladder swab, rectum tissue, gallbladder tissue) were taken for the detection of the nalidixic resistant strain of E. coli O157.

Detection and Enumeration of E. coli O157 Pre-necropsy

Fecal samples were collected on the mornings of sampling days and within one hour a one gram sample from each calf was placed into nine mL of GN-vcc broth and vortexed. For enumeration, 10-1 to 10-4 serial dilutions of fecal suspensions were made in peptone water dilution banks, and 0.1mL of each of the dilutions were spread plated, in triplicate, onto SMAC containing 20 µg/mL naladixic acid (SMAC-nal). Plates were incubated overnight and sorbitol negative colonies were counted and recorded. A maximum of three colonies were plated on blood agar, incubated overnight, and indole and latex agglutination tests performed to ensure that colonies were E. coli O157.

Two additional methods were used to detect calves shedding below the detection limit (<102) of the direct enumeration. The remaining GN-vcc broth and sample was processed according to our standard lab protocol of IMS following a six hour incubation. Colonies that were indole and agglutination positive following the standard protocol, were plated onto SMAC-nal and incubated overnight to confirm the nalidixic acid resistance of the E. coli O157 being shed. Additionally, a selective enrichment technique was also done to detect E. coli O157:H7 if the enumeration plating did not provide positive results. A one mL aliquot of the six hour incubated sample in GN-vcc was transferred into a new tube containing nine mL GN-vcc broth and incubated overnight. If

no sorbitol negative colonies were detected on the direct enumeration platings, 0.1mL from this enriched sample was spread plated onto SMAC-ct-nal and incubated overnight at 37°C. From these plates a maximum of three sorbitol negative colonies were picked, plated on blood agar, and incubated overnight. These colonies were then indole and latex agglutination tested to confirm they were E. coli O157.

Measurement of phagocytic and oxidative burst activities of bovine leukocytes

Blood samples were collected in heparin tubes via jugular venipuncture on days one, 12, and 40 post-final-dexamethasone administration and processed within one hour of collection. Samples were processed and then analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) as described by Flaminio et al (2002).

Detection and Enumeration of E. coli O157 Post-necropsy

Gastrointestinal content samples (rumen, omasum, abomasum, jejunum, ileum, and rectum) were processed according to our standard lab protocol as described above with one gram of contents being used. Tissue swabs (tonsil, cranial lymph node, gall bladder, mesenteric lymph node, peyer's patches, and recto-anal mucosal swab) were placed in three mL GN-vcc broth and processed by the procedure used for fecal samples. Tissue samples (gall bladder and recto-anal mucosa) were cut into approximately one gram pieces, suspended in GN broth, and homogenized for one minute with a tissue homogenizer (Brinkman Instruments, Westbury, NY). The suspension was then processed as described previously.

Statistical Analysis

Data analysis was performed using SAS. A repeated measures analysis of variance with calf nested within group was performed using the log-transformed counts of E. coli O157 shedding for each day as the dependant variable. Individual calf, calves as a group, days post-inoculation, and group by day were repeated. Two data sets were run: the first including shedding values from all days of the study and the second including only days two to 11 post-inoculation. T-tests were performed using calf nested within group and the percent phagocytosis or the percent phagocytosis with oxidative burst as the dependent variable.

Results

All calves tested negative for E. coli O157 in feces prior to oral inoculation. The CBC results (Tables 2.1-2.3) from the dexamethasone treated group from day three post-inoculation showed a marked neutrophilia and a lymphopenia compared to the control group, suggesting that dexamethasone induced immunosuppression was achieved. The CBC results from day 14 post-inoculation showed a resolution in the neutrophilia and the presence of a slight lymphopenia in the dexamethasone treated group. Both the neutrophilia and lymphopenia were resolved in subsequent blood samples (days 25 and 42). There were no statistical differences in phagocytosis and oxidative burst activities between the control and dexamethasone treated groups (data shown in Table 2.4).

The peak shedding level of E. coli O157 by all calves was four days post-inoculation, after which there was a sharp decline in the fecal concentration (Figure 2.2). Individual calves shed at variable levels from day seven post-inoculation until the end of the study. Starting on week two post-inoculation there were animals that were detected as shedding via only enrichment methods. All calves stopped shedding E. coli O157 in their feces by four weeks post-inoculation. Analysis indicated a day by treatment group interaction over the course of the study. Analysis using intervals of days showed that on days four and seven post-inoculation, the dexamethasone treated group of calves shed E. coli O157 at a higher level ($p=0.038$) than the control group. Thereafter, both the dexamethasone treated and control groups shed at approximately the same levels and for the same length of time. All samples collected at necropsy were negative for E. coli O157 (no data shown). All calves were healthy throughout the length of the study.

Discussion

Several studies have used oral inoculation of calves with E. coli O157 to detect duration and level of shedding of the bacteria in feces (Brown, et al., 1997; Cray and Moon, 1995; Sanderson, et al., 1999). As in those studies, none of the calves in this study became clinically ill. The duration of shedding observed in this study is consistent with Cray and Moon's (1995) and Sanderson's (1999) findings with orally inoculated calves as well as with cattle in the field (Besser et al, 1997).

While there have been studies examining the effects of weather, feeding strategies, and strategies of inoculation to examine duration and magnitude of E. coli O157 fecal shedding (Dunn et al, 2004;Cray et al, 1998; Shere et al, 2002), this is the first study to examine the effect immunosuppression has on fecal shedding of E. coli O157. High doses of dexamethasone are commonly used for stress-like immunosuppression in cattle (Anderson et al, 1999) and usage has been associated with reoccurrence of latent infectious bovine rhinotracheatis virus and bovine herpesvirus 2, as well as some blood born parasitic diseases (Roth and Kaeberle, 1982). Clinically, cattle treated with dexamethasone show a decrease in the number of circulating lymphocytes and a dramatic rise in the neutrophil numbers (Anderson, et al., 1999; Burton, et al., 1995; Roth and Kaeberle, 1982). Accompanying the lymphopenia, Anderson et al (1999) have found that IgM concentrations are decreased with dexamethasone injection. The neutrophilia that results from dexamethasone injections is due to the neutrophils' reduced ability to attach to the vascular endothelium and migrate into the tissues to ingest bacteria (Burton et al, 1995). Furthermore, Roth and Kaeberle (1981) found that the neutrophils ability to phagocytose and undergo oxidative metabolism to destroy bacteria are impaired but returned to normal 48 hours post-dexamethasone injection. The calves in this study that received the dexamethasone injection responded with lymphopenia and neutrophilia as adult cattle have in past studies (Anderson, et al., 1999; Roth and Kaeberle, 1981). However, there were no differences in the phagocytosis-oxidation abilities of the neutrophils between the dexamethasone treated and control groups. This may in part be due to the differences in the dosage of dexamethasone given (0.04 mg/kg versus a 0.08mg/kg followed by a 0.25mg/kg doses in those studies). Also, lack of effect may be because neonatal calves were used in our study, whereas Roth and Kaeberle used adult cattle (1981). Studies have shown that dexamethasone's affects last anywhere from two to 12 days post injection (Anderson, et al., 1999; Burton et al, 1995). Since five high end dosages of 0.25mg/kg were given to the calves in this study, a longer duration of immunosuppression was expected. Our CBC data suggest that the calves were immunosuppressed on day three but not on day 14 post-E. coli O157 inoculation. Because no blood samples were taken during this interval, the duration of immunosuppression could not be ascertained.

Data from this study suggest that there may be a relationship between immunosuppression and level of E. coli O157 shedding. Dexamethasone-treated calves shed at higher levels during the days when they would have been immunosuppressed, suggesting there may be a time dependent correlation between dexamethasone immunosuppresion and the concentration of E. coli O157 an animal will shed. It could be surmised that a higher level of E. coli O157 will be shed for as long as dexamethasone suppresses the immune system. If this is true, level or duration of shedding could be impacted by persistent immunosuppression in cattle.

Calves in this study were necropsied four weeks after the last calf stopped shedding E. coli O157 in the feces. Stoffregen et al (2004) cultured O157 in gallbladders after dexamethasone injection of calves but necropsies were performed four days post-E. coli O157 inoculation. Our study provides no support for a site of persistent colonization in the gastrointestinal tract of E. coli O157, which is in agreement with a recent report by Reinstein et al. (2007).

The dexamethasone model used in this study may be comparable to short term immunosuppression caused by stresses common in the cattle industry such as weaning (dietary changes), confinement, and transport. In all cases there is an increase in cortisol levels, although there are not necessarily the same blood cell effects with environmental stressors as there are with dexamethasone injections. While Barham et al (2002) found that shipping steers and heifers on trailers for 30-40minutes caused an increase in the fecal shedding of Salmonella spp, there was no increase in E. coli O157 shedding. However, Bach et al (2004) found that non-preconditioned steer calves that were hauled for 15 hours did have a significantly higher level of E. coli O157 shedding. Another study showed that calves that had feed withheld for 48 hours prior to inoculation with E. coli O157 were more likely to become infected and also shed higher levels of E. coli O157 in their feces (Cray et al, 1998).

Conclusion

In summary, the data from this study suggest that short-term immunosuppressed cattle may shed E. coli O157 at higher levels in their feces, and thus be a significant source of exposure in the farm or feedlot. This environmental fecal contamination could

lead to increased levels of transmission between cattle in the same environment. Once the cattle that are potentially shedding E. coli O157 are transported, they have the possibility of inoculating new environments and cattle. Another perspective to consider is how long term immunosuppression, such as persistent infection with Bovine Viral Diarrhea virus, would potentially effect the long term fecal shedding of E. coli O157. Further research is needed to define the range of immunosuppressive sources and their effects on fecal shedding of E. coli O157 in cattle.

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Table 2.1: Selected CBC results from pre-O157 inoculation and pre-dexamethasone injection.

Calf #	**Segmented neutrophils/μL**	**Band neutrophils/μL**	**Lymphocytes/μL**
Group 1	*Dexamethasone*		
1	3600	0	2800
2	1200	0	3600
3	3600	0	2600
5	4500	0	4100
6	4600	0	1600
10	4600	0	4900
Group 2	*Control*		
4	4300	0	1900
7	2600	0	1800
8	1700	0	2300
9	1100	0	5800
11	1600	0	2100

Table 2.2: Selected CBC results from day three post-O157 inoculation.

Calf #	**Segmented neutrophils/μL**	**Band neutrophils/μL**	**Lymphocytes/μL**
Group 1	*Dexamethasone*		
1	18000	0	980
2	14000	0	1300
3	20000	0	870
5	13000	160	2600
6	11000	400	1900
10	22000	0	980
Group 2	*Control*		
4	2500	0	2200
7	1100	0	3000
8	5500	0	2300
9	720	330	3300
11	1900	0	1600

Table 2.3: Selected CBC results from day 14 post-O157 inoculation.

Calf #	**Segmented neutrophils/μL**	**Band neutrophils/μL**	**Lymphocytes/μL**
Group 1	*Dexamethasone*		
1	3000	0	1100
2	1900	0	1300
3	2300	0	1100
5	1100	0	2700
6	4700	0	2400
10	2400	0	1300
Group 2	*Control*		
4	1700	0	2400
7	3200	0	2100
8	3600	0	2400
9	12000	170	4400
11	760	0	2300

Table 2.4. Phagocytosis only and phagocytosis with oxidative burst results (average ± standard error).

% Phagocytosis	Day 3 post-O157 inoculation	Day 14 post-O157 inoculation
Control Group	25.44 ± 3.99	51.56 ± 3.99
Dexamethasone Group	38.46 + 3.64	43.30 ± 3.64
% Phagocytosis with Oxidative Burst	Day 3 post-O157 inoculation	Day 14 post-O157 inoculation
Control Group	19.23 ± 6.98	15.27 ± 6.98
Dexamethasone Group	26.55 ± 6.37	22.68 ± 6.37

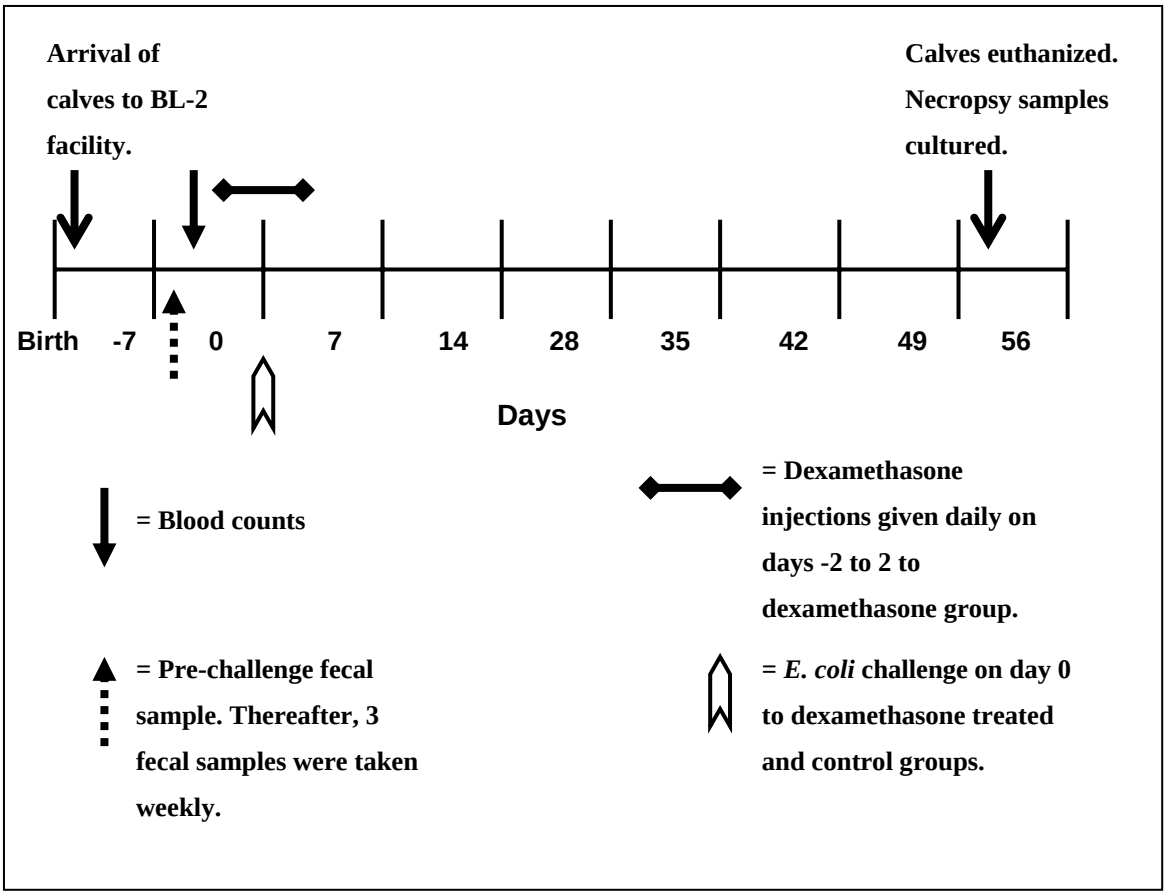

Figure 2.1: Timeline of project details.

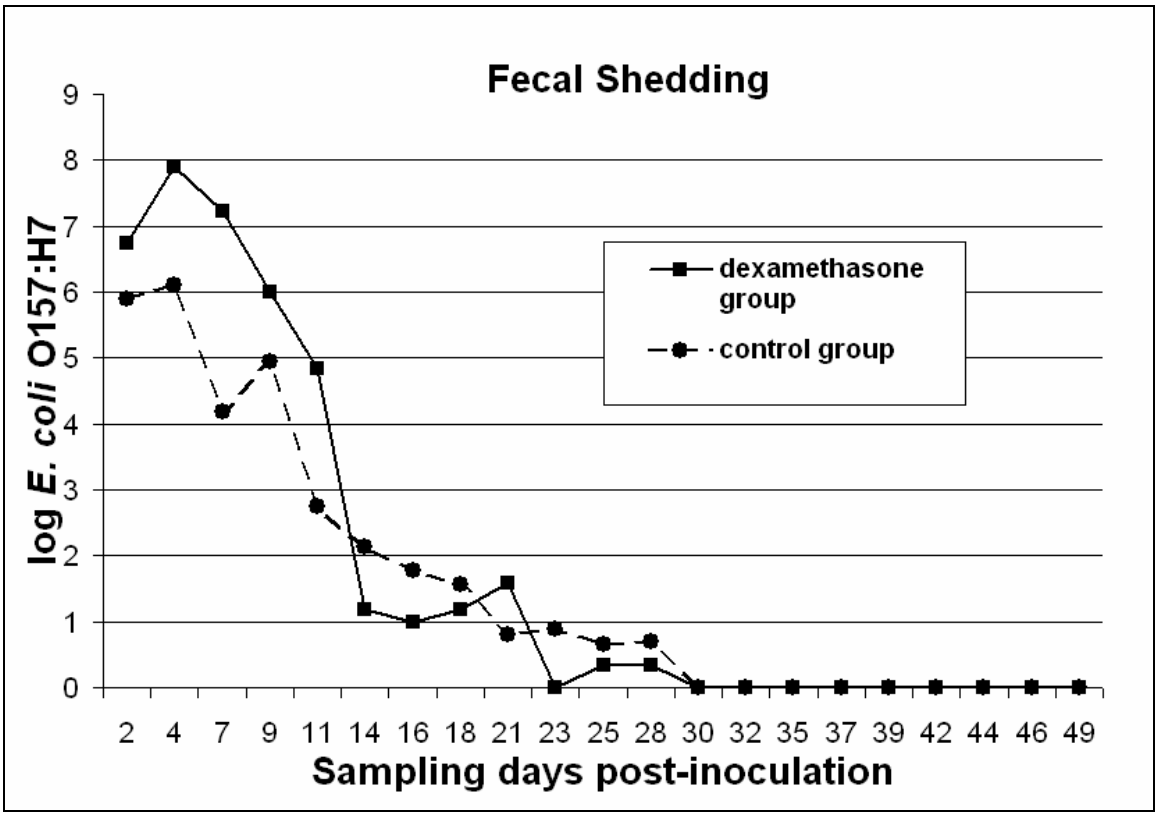

Figure 2: Log_{10} average fecal shedding of *E. coli* O157:H7 from dexamethasone treated and control calves.

CHAPTER 3 - Impact of Persistent Bovine Viral Diarrhea Viral Infection on the Duration and Level of Shedding of *E. coli* O157:H7 in Calves

Abstract

The goal of this study was to determine whether calves immunosuppressed by persistent infection with bovine viral diarrhea virus (BVDV) will shed Escherichia coli O157 at a higher level and for a longer duration than a normal animal. Bovine viral diarrhea is an economically important viral disease in cattle. BVDV causes immunosuppression in the animal, which often leads to secondary infection with other pathogens resulting in severe illness. Nine calves, six to eight week old, persistently infected with non-cytopathic BVDV and eight normal calves obtained from separate cow-calf operations were used in the study. BVDV status, positive or negative, of all calves was confirmed by repeated testing throughout the study. Both groups were orally inoculated with 109 CFU of five nalidixic-acid resistant strains of E. coli O157. All calves were examined daily and fecal samples were collected three times a week for 55 days for detection and enumeration of the nalidixic-acid resistant E. coli O157. Calves were then necropsied and samples from the gastrointestinal tract were taken for the detection of the nalidixic-acid resistant E. coli O157. Data analysis indicated no statistical difference in the concentration of E. coli O157 shed or the duration of shedding between the persistently infected BVDV calves and the control calves throughout the length of the study. Our data suggest that immunosuppression caused by persistent infection with non-cytopathic BVDV infection does not play a role in the level or duration of shedding of E. coli O157:H7 in cattle.

Introduction

Enterohemorrhagic Escherichia coli O157:H7 has emerged as a significant food-borne pathogen over the past two decades. Infected cattle are asymptomatic shedders of the bacteria, but human infection can cause hemorrhagic colitis; which can be more severe in children, the elderly, and other immunocompromised individuals. In the United States, 85-95 percent of severe hemolytic-uremic syndrome in children is caused by E. coli O157:H7 (Armstrong, et al., 1996). Studies have indicated that E. coli O157 is a ubiquitous agent within cattle populations. Beef and beef products have been a significant human source for the pathogen (Hancock, et al, 1998; Galland, et al 2001). However, more recently, fresh vegetables, possibly contaminated with cattle feces, have become the primary source of human infection (MMWR, 2007).

Some researchers suggest that a few animals in a herd may shed E. coli O157 at higher levels and for a longer duration of time compared to other animals in the same herd. These animals have been termed “super shedders.” (Omisakin et al., 2003). These animals may be important in the on-farm epidemiology, ecology, and shedding of E. coli O157 and may also play an important role in pre-harvest food safety. According to modeling data (Matthews, et al., 2006), a small number of “super shedders” may be responsible for the majority of transmission within a farm and thus result in an elevated farm prevalence. Therefore, determining what causes these “super shedders” to shed at higher levels and/or for a longer duration is important.

Bovine viral diarrhea virus (BVDV) is ubiquitous in the cattle population in the U. S. and is known to be immunosuppressive. Cattle may be transiently or persistently infected with BVDV. It has been well documented that BVDV is immunosuppressive in various clinical forms of BVD (Chase, et al., 2004). The objective of this study was to determine whether immunosuppression caused by BVDV, plays a role in the level and duration of fecal shedding of E. coli O157.

Materials and Methods

Figure 3.1 provides a basic time sequence of the study.

Calf Management

Eight 6-8 week old BVD negative calves from a single cow-calf operation and nine immunohistochemistry (IHC) positive BVD calves from two different cow-calf operations were obtained and transported to a BL-2 facility at Kansas State University. BVD negative calves and BVD positive calves were transported separately and were maintained separately throughout the study. Upon arrival to the BL-2 facility, calves were placed into pens in groups; the controls were randomly separated into two pens, and the BVD calves were placed in two pens according to the source they were from. After one week, each calf was housed in an individual pen with individual feed and water buckets and physical contact between calves was prevented. All calves were offered free choice water from day one. Calves were weaned upon arrival and within a week, all calves were consuming grain (dairy calf grower diet from the local cooperative), two flakes alfalfa hay, and free choice grass hay everyday.

Pre-Challenge

Calves were acclimated to the facility for 10 days during which three fecal samples were obtained from the rectum and tested for E. coli O157 (Sanderson et al., 2006) . Briefly a one gram fecal sample was incubated in nine mL of gram negative broth (BD, Franklin Lakes, NJ) with vancomycin (8 g/L), cefixime(0.05 mg/L), and cefsulodin (10 mg/L) (GN-vcc) at 37oC for six hours. The samples underwent manual immunomagnetic separation (IMS), and were plated on Sorbitol MacConkey agar (Becton Dickinson, Franklin, MD) containing cefixime (50 ng/ml) and tellurite (2.5 g/ml) (SMAC-ct) and incubated overnight at 37 oC. Up to six colonies that were morphologically typical of E. coli O157 were streaked on blood agar and incubated overnight. Colonies that were positive on both indole and latex agglutination tests (Oxoid Ltd., Basingstoke, U.K.) were confirmed as E.coli with Rapid API kits (Biomerieux, Hazelwood, MO). Two days prior to inoculation with E. coli O157, jugular vein blood samples were taken from each calf into heparinized blood tubes to provide baseline values for leukocyte activity.

Assessment of BVDV status

The BVD-PI calves were identified in the herds of origin by positive immunohistochemistry testing and then confirmed as persistently infected by virus isolation or a second immunohistochemistry test, with all tests being conducted prior to arrival to the Kansas State University facility. Upon arrival at the Kansas State University BL-2 facility, ear notches were obtained on all the control calves for a BVD immunohistochemistry test and jugular blood samples were taken from all calves for virus isolation. During the course of the study, three additional virus isolations were performed on days 5-6, 19-20, and 33-34 post-E. coli O157 inoculation, (control calves were sampled the first day and PI calves were sampled the second day) to confirm the negative BVD status of the control calves, and persistent status of the BVD calves.

Calf Challenge and Sampling Schedule

All calves were inoculated with 109 colony-forming units of E. coli O157 using an esophageal feeding tube. The inoculum was prepared from a frozen stock of five strains adapted to nalidixic acid (20 µg/ml) (01-2-10004, 01-2-12329, 01-2-10530, 01-2-10561, and 01-2-8970). Each strain was grown individually, pooled together on the day of inoculation, and diluted with water to inoculate the calves. Calves were examined daily and fecal samples were collected three times a week (Monday, Wednesday, and Friday) for detection and enumeration of the nalidixic acid resistant E. coli O157. Blood samples from the jugular vein were collected from all calves into heparinized and regular blood tubes on days 5-6, 19-20, 33-34, and 54-55 post-E. coli O157 inoculation: PI calves were sampled the first day and control calves were sampled the second day. Eight weeks post- E. coli O157 inoculation, nine calves (five BVD-PIs and four controls) were euthanized with Fatal-Plus® and necropsied. The remaining eight calves (four BVD-PIs and four controls) were euthanized and necropsied four days afterwards.

Detection and Enumeration of E. coli O157 Pre-necropsy

Fecal samples were collected on the morning of the sampling days and a one gram sample from each calf was placed into nine mL of GN-vcc broth within one hour of collection and the tubes were vortexed. Samples were enumerated by making 10-1 to 10-4 serial dilutions of fecal suspensions in peptone water dilution banks, and 0.1mL of each

of the dilutions were spread plated, in triplicate, onto SMAC containing 20 µg/mL naladixic acid (SMAC-nal). Morphologically typical, sorbitol negative colonies on enumeration plates were counted and recorded after overnight incubation. A maximum of three colonies were plated on blood agar, incubated overnight, and indole and latex agglutination tests performed to ensure that colonies were E. coli O157.

Two additional methods were used to detect calves shedding below the detection limit (<102) of the direct enumeration. First, the remaining sample in the GN-vcc broth was incubated for six hours at 37°C and then underwent IMS. Resulting colonies that were indole and agglutination positive were plated onto SMAC-nal and incubated overnight to confirm the nalidixic acid resistance. Secondly, a selective enrichment technique was done to detect E. coli O157 if the enumeration plating did not provide positive results. For this, a one mL aliquot of the six hour incubated sample in GN-vcc was transferred into a new tube containing nine mL GN-vcc broth and again incubated overnight at 37°C. If no sorbitol negative colonies were detected on the direct enumeration platings, a 0.1mL aliquot from this enriched sample was spread plated onto SMAC-ct-nal and incubated overnight at 37°C. From these plates a maximum of three sorbitol negative colonies were picked, plated on blood agar, and incubated overnight. These colonies were then indole and latex agglutination tested to confirm they were E. coli O157.

Measurement of phagocytic and oxidative burst activities of bovine leukocytes and Immunophenotyping

Blood samples were collected in heparin tubes via jugular venipuncture on days 2-3 prior to E. coli O157 inoculation and on days 19-20 and 33-34 post-E. coli O157 inoculation (controls sampled the first day and PIs sampled the second day) and processed within one hour of collection. Phagocytic and oxidative burst activities were analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) as described by Flaminio et al (2002). Immunophenotyping of leukocytes was done using monocolonal antibodies (VMRD, Pullman, WA, USA) specific for CD2, CD4, CD18, CD14, CD21, CD44, CD62L, MHC I, MHC II, and TCR. Briefly, whole blood samples were centrifuged at 3400rpm for six minutes. The plasma layer was aspirated and

discarded. Ammonium chloride was added to the remaining pellet and allowed to sit for five to ten minutes to allow the erythrocytes to lyse. Samples were then centrifuged at 3400rpm for three minutes and the supernatant was discarded. The leukocyte pellet was then washed with 0.1M PBS, centrifuged at 3400rpm for three minutes, the supernatant was discarded, and the pellet was re-suspended in PBS to obtain a leukocyte concentration of 1x107 cells/mL. A 100µL aliquot of cells and 100µL of the appropriate monoclonal antibody were mixed and incubated for twenty minutes in the dark at two to six degrees Celsius. The samples were then washed in three mL of PBS, centrifuged on high for three minutes, and the supernatant was discarded. A 100µL aliquot of the fluorescent conjugated secondary antibody was added, mixed, and the samples were placed in the dark for ten minutes at two to six degrees Celsius. The samples were washed in three mL of PBS, centrifuged at high speed for three minutes, and the supernatant was discarded. The cell pellet was re-suspended with 250mL of PBS and the samples were analyzed using the FASCcan flow cytometer.

Detection and Enumeration of E. coli O157 in Samples Collected at Necropsy

At necropsy, one gram samples of gastrointestinal content from the rumen, omasum, abomasum, jejunum, ileum, and rectum were processed according to our standard lab protocol as described above. Tissue swabs from the tonsil, cranial lymph node, gall bladder, mesenteric lymph node, peyers patches, and recto-anal mucosa were placed in three ml GN-vcc broth and then processed by the same procedure used for previously descrived fecal samples. Tissue samples (gall bladder and recto-anal mucosa) were cut into approximately one gram pieces, suspended in nine mL GN-vcc broth, and homogenized for one minute with a tissue homogenizer (Brinkman Instruments, Westbury, NY). The suspension was then processed as described previously.

Statistical Analysis

Using a commercially available statistical software (SAS version 8.0, Cary, NC), a repeated measures analysis of variance with calf nested within group was performed on the log of the enumerated counts of E. coli O157 fecal shedding for each day. Individual calf, group, day post-inoculation, and group by day were the repeated measures. Phagocytic and oxidative burst activity and immunophenotyping data was also evaluated

using a repeated measures analysis of variance with calf nested within group. Individual calf, group, day pre- or post-inoculation, and group by day were repeated measures. Due to the small number of samples, a p-value less than or equal to 0.0047 was designated for significance using the Bonferroni correction (0.05/12 antigens = 0.0047).

Results

All calves were healthy throughout the length of the study. All control calves were negative for BVDV by IHC and virus isolation at all sampling times throughout the study and all BVD-PI calves remained virus isolation positive at all sampling times. Two control calves tested positive for E. coli O157 in the first pre-challenge fecal collection but tested negative on days seven and two prior to inoculation. All other calves tested negative for E. coli O157 in feces prior to oral inoculation. The peak shedding level of E. coli O157 by all calves was one day post-inoculation, after which there was a sharp decline in the fecal concentration (Figure 3.2). Individual calves shed at variable levels throughout the study. Overall both the control and BVD groups shed at approximately the same levels and for the same length of time, indicating no significant difference in shedding levels between the two groups. One and a half weeks post-inoculation there were animals that were culture positive only via enrichment methods. Nine calves (four controls and five PIs) had negative fecal samples for at least ten days prior to necropsy. Six calves (three controls and three PIs) were still detected as shedding E. coli O157 in their feces three days prior to necropsy. Of these six calves, only the three control calves tested positive for E. coli O157 at necropsy. The positive samples were collected from the cecal, colon, and rectal contents, and rectal tissue. The phagocytosis, oxidative burst, and immunophenotyping analysis showed no difference between the leukocyte activities of the BVD-PI calves and the control calves (Tables 3.1-3.6).

Discussion

Even though nine of the calves in this study were persistently infected with BVDV, none of the calves became clinically ill during the course of the study. This is

consistent with previous studies that have used oral inoculation of calves with E. coli O157 to detect duration and level of shedding of the organism in feces (Brown, et al., 1997; Cray and Moon, 1995; Sanderson, et al., 1999). The duration of shedding observed in this study is consistent with data from both Cray and Moon (1995) and Sanderson et al (1999) in orally inoculated calves as well as with cattle in the field (Besser et al, 1997).

Causes of immunosuppression in cattle include stress, such as weaning (dietary changes), confinement, and transport. In all cases there is an increase in cortisol levels, which may alter leukocyte counts in the animal. Barham et al (2002) found that short term shipping of steers and heifers on trailers (30-40minutes) caused an increase in the fecal shedding of Salmonella spp, but no increase in E. coli O157 fecal shedding. Bach et al (2004) studied stress in relation to fecal shedding of E. coli O157 in four groups of calves: the first and second groups were pre-conditioned (vaccinated and weaned at four or two weeks prior to transport) prior to either a three or 15 hour transport to the feedlot, and the third and fourth groups (non-preconditioned) were weaned one day prior to either a three or 15 hour transport and were vaccinated after they arrived at the feedlot. Of all four groups, only the non-preconditioned steer calves hauled for 15 hours shed E. coli O157 had a significantly higher fecal prevalence. This study suggested that dividing the pre-harvest stresses over a period of time, rather than compounding them at one time, may reduce shedding levels of E. coli O157. Finally, Cray et al (1998) showed that calves that had feed withheld for 48 hours prior to inoculation with E. coli O157 were more likely to become infected and also shed higher levels of E. coli O157 in their feces. As a whole, these studies suggest that stress events may change the prevalence and possibly the shedding dynamics of E. coli O157. However, whether these changes were due to general effects on microbial ecology of the gut or the effect of immunosuppression is unknown.

There have been studies examining the effects of weather, feeding strategies, and strategies of inoculation to examine duration and magnitude of E. coli O157 fecal shedding (Dunn et al, 2004; Cray et al, 1998; Shere et al, 2002); however, this is the first study to examine the effect of BVDV persistent infection may have on fecal shedding of E. coli O157. If a cow is infected with a noncytopathic BVD virus between days 45 and 125 of gestation, the virus may infect the fetus and result in the birth of a calf persistently

infected (PI) with the noncytopathic BVD virus (Baker, 1995). These cattle in turn shed large numbers of the virus into their environment and are therefore a significant threat to spread of the virus to naïve cattle (Houe, 1999). Persistently infected cattle may have decreased weight gain and decreased growth, but may also appear clinically normal. Additionally, data indicate that PI cattle have decreased neutrophil and lymphocyte functions (Brown, et al, 1991 and Chase, et al, 2004). In healthy calves inoculated with a non-cytopathic strain of BVD, Ellis et al (1988) also found a decrease in the number of circulating B and T lymphocytes. Cattle with BVDV immunosuppression often succumb to secondary bacterial respiratory infections causing substantial economic loss (Campbell, 2004). Brewoo et al (2007) found that there was no difference in the phagocytic activities and leukocyte profiles between persistently infected adult BVD cattle and controls. According to our CBC and flow cytometric data, there was no significant difference in leukocyte numbers, phagocytic or oxidative burst activity, or expression of antigens on leukocytes between BVD-PI calves and control calves, which is in agreement with the Brewoo paper.

Our data does not support a role for BVD-PI infection in increasing either the level or duration of shedding of E. coli O157 in cattle. However, other alternative effects may exist. BVDV also causes transient infections in cattle, which are more common than persistent infections. Data shows that healthy calves experimentally infected with a field isolate of BVD had a significant decrease in the number of neutrophils, lymphocytes, and monocytes 3-5 days post inoculation compared to control calves (Archambault, et al, 2000). Another study showed that calves inoculated with a non-cytopathic strain of BVDV had a significant decrease in the number of circulating platelets (Bezek, et al, 1994). While it has been documented that PI cattle are immunosuprressed (Potgieter, 1995), the mechanism causing the immunusuppression in poorly understood. More research needs to be done to determine the difference in the immunosuppressive factors between acutely infected and persistently infected BVD cattle.

Nine calves in this study were necropsied eleven days after the last calf stopped shedding E. coli O157 in the feces. Of the remaining eight calves, six were still shedding E. coli O157 three days prior to necropsy. Of those six, only three had O157 cultured from the necropsy samples. These positive sites included cecal, colon, and rectal

contents, and homogenized rectal tissue. Stoffregen et al (2004) cultured O157 in gallbladders of calves given dexamethasone injections and necropsied four days after E. coli O157 inoculation. Our study provides no support for a site of persistent colonization of E. coli O157 in the gallbladder, which is in agreement with a recent report by Reinstein et al. (2007).

Conclusion

In summary, the data from this study do not confirm that calves persistently infected with BVDV alter the duration or level of fecal shedding of E. coli O157. However, further research is needed to define the range of immunosuppressive factors and their effects in viral inection on fecal shedding of E. coli O157 in cattle.

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Table 3.1. Results of percent phagocytosis only and percent phagocytosis with oxidative burst (average ± standard error). Days are in reference to *E. coli* O157 inoculation (controls sampled the first day and BVD-PIs sampled the second day).

% Phagocytosis	**Days -3 and -2**	**Days 19 and 20**	**Days 33 and 34**
Control Group	33.72 ± 4.56	25.43 ± 4.56	30.60 ± 4.56
BVD-PI Group	36.74 ± 4.30	21.76 ± 4.30	18.03 + 4.30
% Phag-Ox			
Control Group	25.45 ± 3.55	30.71 ± 3.55	44.40 ±3.55
BVD-PI Group	22.89 ± 3.35	36.51 ± 3.35	38.06 ± 3.35

Table 3.2. P-values for Phagocytosis and oxidative burst data. ($p < 0.0047$ is significant)

	Whole data set	**Days -3and-2**	**Days 19and20**	**Days 33and34**
%Phagocytosis	0.27	0.63	0.21	0.05
% Phag-Ox	0.59	0.60	0.24	0.41

Table 3.3. Percentage of calves' lymphocytic cells (± standard error with 95% CI) reacting with monoclonal antibody specific to antigens tested. (Days are in reference to day of *E. coli* O157 inoculation with controls sampled first day and BVD PIs sampled second day.

Antigen	Group	Day -3and-2	Day 19and20	Day 33and34
CD 2	Control	25.13±3.19	34.44±3.19	42.02±3.19
	PIBVD	28.62±3.01	38.11±3.01	38.64±3.01
CD4	Control	7.81±2.19	17.23±2.19	20.85±2.19
	PIBVD	9.97±2.07	23.23±2.07	22.53±2.07
CD8	Control	12.43±1.96	11.74±1.96	24.44±1.96
	PIBVD	13.63±1.85	14.38±1.85	18.93±1.85
CD11a	Control	33.02±4.20	1.95±4.20	28.77±4.20
	PIBVD	22.05±3.96	4.54±3.96	24.26±3.96
CD14	Control	14.22±2.71	5.23±2.71	15.87±2.71
	PIBVD	10.28±2.55	7.99±2.55	18.75±2.55
CD21	Control	2.47±4.35	1.14±4.35	9.36±4.35
	PIBVD	3.23±4.10	13.25±4.10	6.74±4.10
CD44	Control	52.38±4.12	56.13±4.12	73.28±4.12
	PIBVD	40.90±3.89	58.51±3.89	67.83±3.89

Antigen	Group	Day -3and-2	Day 19and20	Day 33and34
CD62L	Control	47.58±2.70	69.97±2.70	71.64±2.70
	PIBVD	45.15±2.55	63.79±2.55	68.47±2.55
MHCI	Control	89.40±3.48	98.33±3.48	99.16±3.48
	PIBVD	96.49±3.28	90.47±3.28	98.05±3.28
MHCII	Control	46.68±4.68	18.21±4.68	48.99±4.68
	PIBVD	60.00±4.41	29.33±4.41	33.25±4.41
TCR	Control	8.28±2.47	31.3±2.47	33.67±2.47
	PIBVD	8.56±2.47	22.56±2.47	25.73±2.47
IL2R	Control	6.31±3.50	1.92±3.50	12.38±3.50
	PIBVD	5.14±3.29	10.83±3.29	9.54±3.29

Table 3.4. P-values for percentages given in Table 3.3. (Days are in reference to day of *E. coli* O157 inoculation) $p < 0.0047$ is significant.

Antigen	Whole data set	Day -2and-3	Day 19and20	Day 33and34
CD2	0.72	0.43	0.41	0.47
CD4	0.17	0.48	0.05	0.58
CD8	0.76	0.66	0.33	0.05
CD11a	0.20	0.06	0.66	0.44
CD14	0.80	0.30	0.48	0.44
CD21	0.44	0.89	0.05	0.66
CD44	0.24	0.05	0.68	0.34
CD62L	0.06	0.02	0.10	0.40
MHCI	0.82	0.15	0.11	0.82
MHCII	0.57	0.05	0.09	0.02
TCR	0.05	0.94	0.02	0.03
IL 2R	0.55	0.81	0.07	0.56

Table 3.5. Geometric mean of fluorescence intensity of calves lymphocytic cells (± standard error with 95% CI). (This is proportionate to the number of binding sites on the cells for the antibody. (Days are in reference to day of *E. coli* O157 inoculation, with controls sampled first day and BVD PIs sampled second day.)

Antigen	Group	Day -3and-2	Day 19and20	Day 33and34
CD 2	Control	316.97±17.37	293.95±17.37	435.07±17.37
	PIBVD	343.11±16.38	330.91±16.38	397.20±16.38
CD4	Control	263.55±15.21	190.70±15.21	296.62±15.21
	PIBVD	267.24±14.33	231.09±14.33	278.48±14.33
CD8	Control	362.14±27.64	449.00±27.64	509.18±27.64
	PIBVD	337.70±26.05	418.57±26.05	445.92±26.05
CD11a	Control	534.00±74.61	290.33±74.61	368.39±74.61
	PIBVD	355.49±70.34	298.13±70.34	321.04±70.34
CD14	Control	373.27±21.64	322.39±21.64	381.18±21.64
	PIBVD	414.38±20.40	292.41±20.40	367.64±20.40
CD21	Control	472.89±67.42	334.70±67.42	336.77±67.42
	PIBVD	547.72±63.57	356.63±63.57	338.04±63.57
CD44	Control	333.80±13.78	330.16±13.78	416.16±13.78
	PIBVD	324.36±12.99	291.12±12.99	374.05±12.99

Antigen	Group	Day -3and-2	Day 19and20	Day 33and34
CD62L	Control	412.43±23.92	388.04±23.92	573.67±23.92
	PIBVD	362.68±22.55	423.91±22.55	508.09±22.55
MHCI	Control	2240.11±287.57	1396.53±287.57	3032.79 ±287.57
	PIBVD	2451.86±271.12	1649.42±271.12	2511.28 ±271.12
MHCII	Control	903.40±79.43	453.51±79.43	969.22±79.43
	PIBVD	1112.70±74.88	453.42±74.88	957.54±74.88
TCR	Control	376.82±29.57	369.25±29.57	522.04±29.57
	PIBVD	361.24±27.88	338.88±27.88	523.25±27.88
IL2R	Control	394.57±18.44	267.86±18.44	301.16±18.44
	PIBVD	401.14±17.38	292.44±17.38	317.71±17.38

Table 3.6. P-values for percentages given in Table 3.5. (Days are in reference to day of *E. coli* O157 inoculation) $p < 0.0047$ is significant.

Antigen	Whole data set	Day -2and-3	Day 19and20	Day 33and34
CD2	0.54	0.23	0.13	0.12
CD4	0.54	0.86	0.06	0.39
CD8	0.18	0.52	0.43	0.10
CD11a	0.24	0.09	0.94	0.65
CD14	0.97	0.18	0.32	0.65
CD21	0.53	0.42	0.81	0.99
CD44	0.77	0.62	0.09	0.04
CD62L	0.19	0.14	0.28	0.05
MHCI	0.21	0.04	0.53	0.20
MHCII	0.34	0.05	1.0	0.51
TCR	0.58	0.70	0.46	0.98
IL 2R	0.41	0.80	0.34	0.52

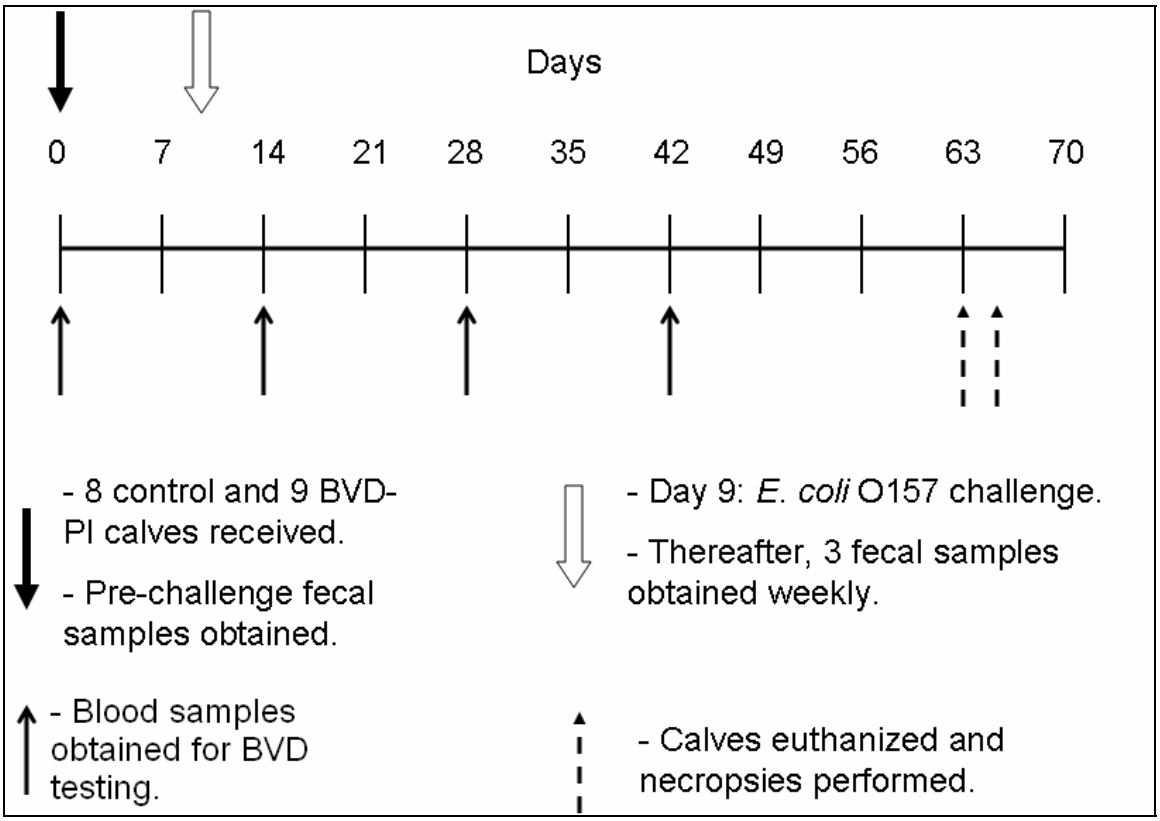

Figure 3.1: Timeline of project details.

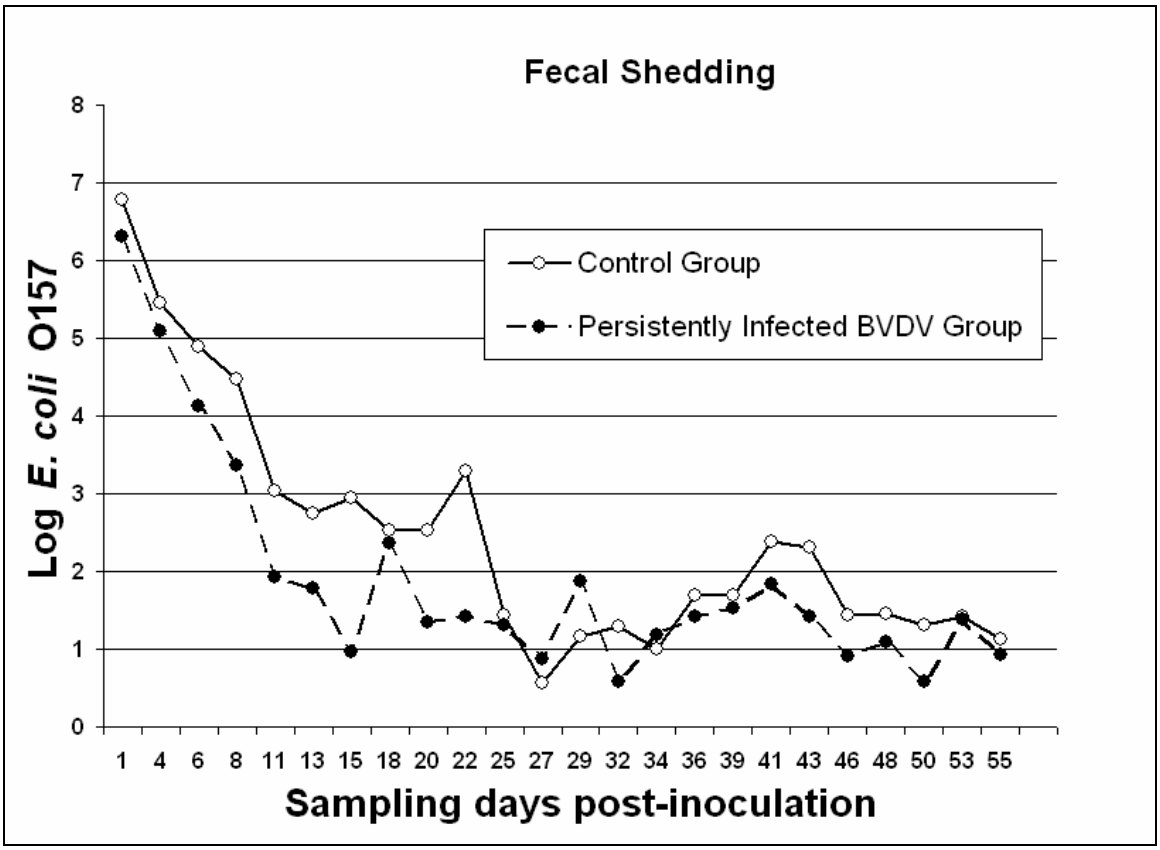

Figure 3.2: Log_{10} average fecal shedding of *E. coli* O157:H7 from control and BVD-PI calves.